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The alteration of the HER-2/*neu* gene has been shown to correlate with a poor prognosis in those patients whose tumors contain it. This has led to studies from our laboratory indicate that it may play a role in the pathogenesis of the disease for some patients. Given that the HER-2/*neu* gene encodes a growth factor receptor found on the membrane of tumor cells and given its potential role in the pathogenesis of some human breast cancers, it is a logical target for the development of new therapeutic approaches directed at this alteration. Studies with monoclonal antibodies directed against the extra cellular domain of the receptor indicate that many may have significant growth inhibitory properties. Recently ligands have been identified which interact either directly or indirectly with the human HER-2/*neu* receptor, however little is known about the biologic effects of these molecules. There is some controversy as to whether the ligands mediate growth stimulatory or growth inhibitory effects or both. A greater understanding about the biologic effects of HER-2/*neu* overexpression as well as the impact of agonists and antagonists to the receptor will be required to fully therapeutically exploit this gene alteration in human breast cancer. Finally, little is know about the biologic effects of other molecular alterations which may occur in combination with HER-2/*neu* expression such changes in the estrogen receptor. The purpose of this proposal is to expand our knowledge base regarding the role of this critical gene in human breast cancer and to determine how to exploit this information clinically.

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PROGRESS REPORT

DAMD17-94-J-4118

BIOLOGIC EFFECTS OF HER-2/*neu* GENE OVEREXPRESSION AND AGONISTS AND ANTAGONISTS TO THE RECEPTOR IN HUMAN BREAST CANCER

PRINCIPAL INVESTIGATOR: Dennis J. Slamon, M.D., Ph.D.

The introductory section as well as the background material for the progress report for this funded proposal remain largely the same as stated in the initial application. Since these sections are required for the progress report, they will be restated here.

INTRODUCTION

The HER-2/*neu* gene is amplified in 25-30% of human breast cancers. When the alteration was examined for association with clinical parameters, it was found that gene amplification was associated with a poor prognosis; i.e. those women whose tumor contained the alteration had a shorter disease-free and overall survival. This association was initially disputed, but a large number of subsequent studies have now confirmed it. Moreover, the prognostic association between HER-2/*neu* alteration and disease outcome is seen in both node positive and node negative breast cancer. This literature and the controversy surrounding the association has been extensively reviewed recently. The bulk of the published data now clearly support the prognostic significance of HER/*neu* overexpression in breast cancer. However, all of the reviewed studies were retrospective analyses of archival cohorts of specimens. Most recently the first prospective study, consisting of 1056 primary human breast cancers, was completed and published and this study also confirmed the prognostic significance of HER-2/*neu* alteration in both node negative and node positive disease . Taken together, these data confirm the prognostic significance of HER-2/*neu* amplification/overexpression in human breast cancer.

There are at least two possible explanations for the association between HER-2/*neu* amplification/overexpression and poor clinical outcome; a) the alteration serves as a genetic marker for a poor outcome but plays no role in causing it; i.e. a useful prognostic epiphenomenon; or alternatively b) the alteration is associated with a poor outcome because it plays a direct role in the pathogenesis of such an outcome. There is circumstantial evidence which supports the latter possibility. These data include the following: 1) the mutated rat *neu* gene can act as a dominant transforming oncogene, underscoring its oncogenic potential and NIH 3T3 cells transfected with the gene are not only transformed *in vitro*, but are tumorigenic in the nude mouse ; 2) monoclonal antibodies directed against the extracellular domain of the rat *neu* gene will inhibit transformation *in vitro* as well as tumorigenicity *in vivo*; 3) studies to develop transgenic mice using the mutated rat gene under the control of an MMTV promoter, demonstrate that these mice develop breast cancer at three months of age; and finally, 5) studies using a transfected human HER-2/*neu* gene in NIH 3T3 cells demonstrate that it too will transform the cells and that higher levels of expression result in greater transforming efficiency, and greater tumorigenicity of the cells . In composite, these data clearly show the ability of the HER-2/*neu* gene to mediate transformation *in vitro* and tumorigenicity *in vivo*.

Each of these lines of circumstantial evidence, however, have shortcomings with regards to implicating the HER-2/*neu* gene in the pathogenesis of human breast cancer. The first line of evidence proved that the rat *neu* gene could be oncogenic; however, sequence analysis showed the gene to have a point mutation in the transmembrane domain. To date, no such mutation has been identified in the human HER-2/*neu* gene. Instead the alteration found in human breast cancer is amplification and overexpression of the normal gene. In addition, the

mutated rat *neu* gene induces neuroglioblastomas and not breast cancer in the animals. The monoclonal antibody studies were equally convincing that the mutated rat gene could have a role in transformation of neural tissue but, again, these antisera were not directed against the human protein nor were they used in altering the phenotype of human breast carcinoma cells overexpressing a normal, non-mutated, human HER-2/*neu* gene. The transgenic mouse studies were particularly compelling in showing that alterations in the rat *neu* gene could result in the development of breast carcinoma but, again, the study had used the mutated rat gene (48). Lastly, the data demonstrating that an overexpressed, non-mutated human HER-2/*neu* gene could transform NIH 3T3 cells proved that the human gene was oncogenic *in vitro* , but the experiments again used NIH 3T3 cells, not human breast cancer or breast epithelial cells. In addition, the levels of overexpression in these studies were far in excess of what is seen in most human breast cancer specimens in nature. To circumvent some of the concerns raised with the experiments utilizing the rat *neu* gene or murine cells (NIH-3T3), we recently designed a series of experiments to introduce the human gene into human breast cancer cells as well as non-transformed immortalized and normal non-immortalized human breast epithelial cells. Important in the studies is to mimic as closely as possible the alteration seen in human tumors and then determine the biologic effects (if any) of this alteration. Central to these studies was the use of the human gene in human cells and the objective to hold the levels of overexpression at or below those seen in actual human tumors; i.e. not to exceed levels found in primary and/or metastatic tumors in nature. These studies have not yet been published but have now been completed as part of this grant support. They are presented as preliminary data in the current report. We feel that they demonstrate our ability to conduct the studies detailed in this application.

In addition we wish to examine the effects that an additional alteration, mutation of the p53 gene, may have on cells overexpressing the HER-2/*neu* gene. There is considerable evidence that the conversion of a normal cell into a malignant cell is a multistep process which may involve the alteration of more than one if not several genes. Mutation of the p53 gene is one of the most common genetic alterations found in human malignancies and is frequently found in breast cancer. The mutation rate in breast cancer is reported to be from a low of 14% to a high of 58% with the most frequently reported rate being between 25-30% of all cases. This incidence makes p53 alteration a potentially important mutation in the pathogenesis of human breast cancer. Similar to the data with HER-2/*neu*, this concept is circumstantially supported by data indicating that p53 mutation is associated with aggressive subtypes of the disease and/or a poor prognosis. The concept gains further support with experimental data demonstrating that introduction of a wild type p53 gene into a human breast cancer cell line containing a mutant gene will suppress the transformed phenotype. Data are now accumulating which indicate that alterations in the p53 gene are frequently associated with HER-2/*neu* alterations . This combination of mutations may be very important in the pathogenesis of some human breast cancers. The HER-2/*neu* overexpressing cell lines already developed as well as those proposed to be developed as part of this application should be useful in addressing this issue.

BODY OF REPORT

Specific Aim I - To further develop a series of human breast epithelial and cancer cell lines containing defined alterations in expression of the human HER-2/*neu* gene.

This specific aim was largely accomplished in the first nine-twelve months of funding for this project (11/94 - 7/95). The methods used to achieve the goals of this Specific Aim involve the introduction of a full length human HER-2/*neu* c-DNA into a series of human breast epithelial cell lines representing normal breast epithelial cells, immortalized but non-transformed breast epithelial cells and breast cancer cells. The cell lines detailed in the proposal, i.e. T47D, MDA-MB-231, MDA-MB-435, BT-20 and BT-483 have all been successfully transfected and engineered to overexpress the HER-2/*neu* gene. These transfectants have been characterized for stable HER-2/*neu* overexpression and all appear to have this feature (at least at 6-months

of follow-up). The biologic characterization of these cells has been similar to those studies presented in the preliminary data in the initial proposal, i.e. ^{3}H-thymidine incorporation, cell growth (*in vitro)* anchorage independent growth and tumorigenicity. In all assays the data for the newly established engineered cells are similar to the data for the MCF-7, B5 and HBL-100 cell lines. DNA synthetic rate increases significantly as does cell growth, anchorage independent growth and tumorigenicity. These data are important in that they demonstrate that the biologic effects of HER-2/*neu* overexpression seen in the MCF-7, B5 and HBL-100 cell lines are not restricted to just those cell lines but can also be achieved in all of the breast cancer cell lines evaluated. These findings lend substantial credence to the concept that overexpression of the HER-2/*neu* gene plays an important pathogenic role in the aggressive biologic behavior of those cells and tumors which contain it. More recent studies using some of these cell lines has lead to insights into the clinical observation that human breast cancers which overexpress the HER-2/*neu* receptor tend to be estrogen receptor negative. Studies performed in our laboratory and, in part, supported by the grant, have demonstrated a potentially important direct interaction between activation of the HER-2/*neu* receptor and down regulation of the estrogen receptor. This is a critical area of research currently as there are developing data indicating a relationship between HER-2/*neu* overexpression and estrogen receptor negativity and tamoxifen resistance. This research has been expanded as part of this proposal in Specific Aim 4. We have also used these cell lines to study the effects of HER-2/*neu* overexpression on expression of molecules believed to be involved in adhesion of cells to other cells as well as stroma and matrix elements in various tissues. The rationale behind this approach is to evaluate the potential mechanism for the observation that HER-2/*neu* over-expressing cells have an increased metastatic potential in in vivo models. Using the transfected HBL-100 cells for which we have a number of clones which range in HER-2/*neu* expression from normal levels to levels of overexpression seen in breast cancer specimens with amplification of the gene, we have determined that there are definite changes which occur in ß-catenin, E-cadherin, ICAM-1, integrins -$\alpha\beta$ 5, $\beta\alpha$ 6 and B1 as well as FAK, depending on HER-2 levels (Figs A-G)

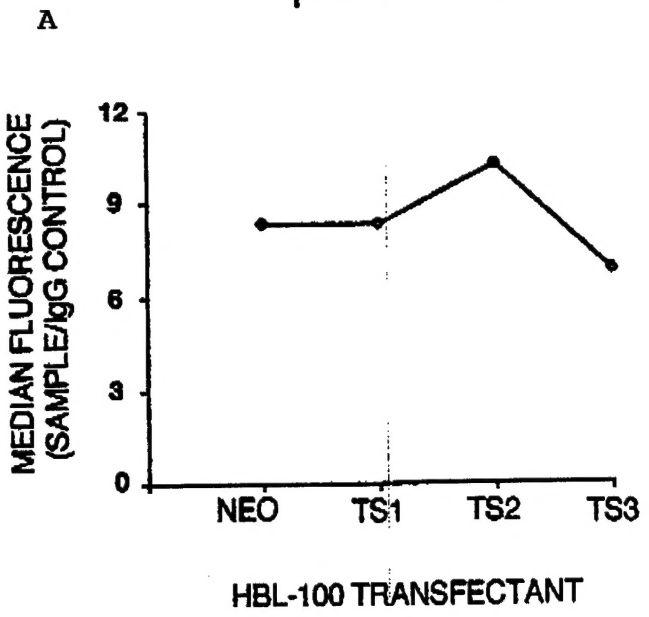
Specific Aim II - To assess the biologic effects of agonists, i.e. the heregulin and *neu* differentiation factor ligands as well as an antagonist, i.e. a monoclonal antibody, to the HER-2/*neu* reception on human breast cells, *in vitro.*

The objective of this Specific Aim is to assess the biologic effects of HER-2/*neu* agonists and antagonists on breast cancer cells with defined levels of HER-2/*neu* expression *in vitro.* The methods for this aim are detailed in the initial proposal and will not be restated here. As stated in last years progress report we determined that heregulin was able to consistently elicit a growth stimulatory effect on cells with HER-2/*neu* overexpression. This effect was measured by ^{3}H-thymidine incorporation, cell counts in proliferation assays, clonogenicity on plastic (anchorage-dependent growth) and cloning efficiency in soft agar (anchorage-independent growth.)

We have now asked the questions, of if and how overexpression of HER-2/*neu* effects expression of the other membranes of the types I receptor tyrosine kinase family, i.e. HER-1, HER -3 and HER -4. This is important because signaling through the HER -2 pathway is linked to heterodimerization of these various family members.

To determine the relative effect of expression of the various members of the RTK I family on HRG response, a modified ELISA was used to quantitate the HER-1, HER-2, HER-3 and HER-4 receptor content of the various cell lines used in the *in vitro* and *in vivo* studies. The sensitivity of this ELISA was validated both by comparison with specific receptor content as determined by Scatchard analysis in a number of the cell lines as well as by quantitative comparison of the HER-2/neu expression levels in SK-BR-3 and MCF-7 cells. HER-2/*neu* expression levels have previously been shown to be 128 times higher in SK-BR-3 compared to MCF-7 cells by RNA analysis. The ELISA data from the current study demonstrates a 129 fold increase in protein content when comparing these two cell lines which is almost identical to the published results and validating the ELISA results. In non-overexpressing cells HER-2/*neu* transfection results in cell lines with 10-100 times more HER-2 receptors/cell compared to matched control cells. This

A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TS2
TS3
HBL-100 TRANSFECTANT

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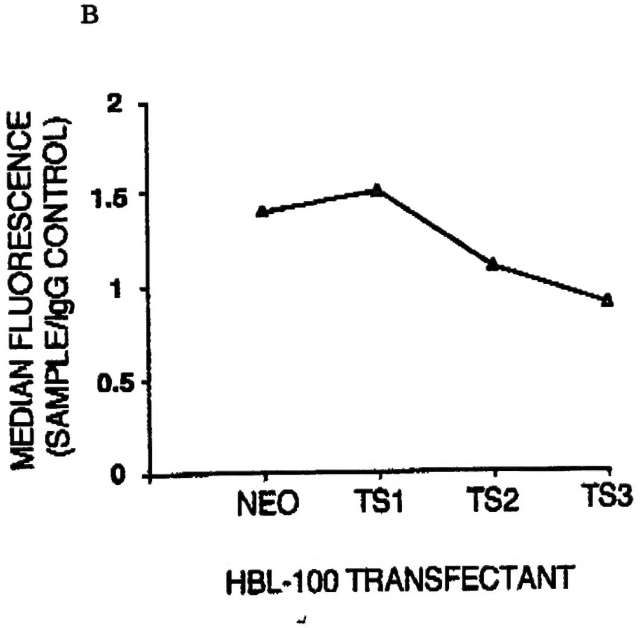
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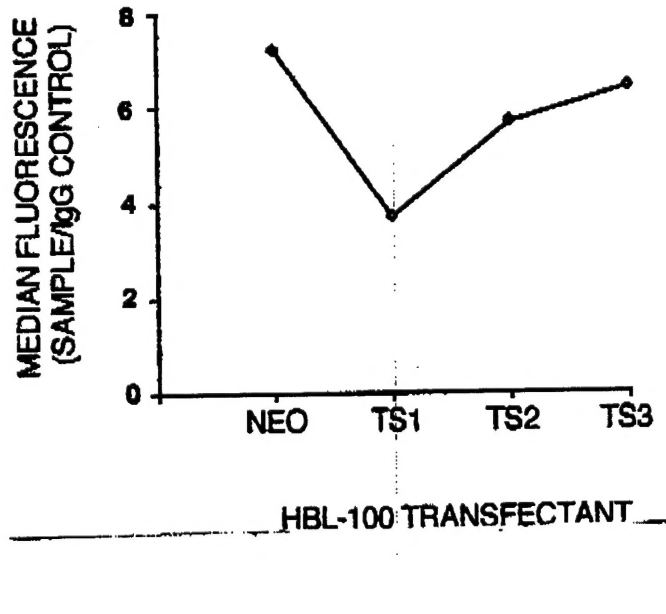
C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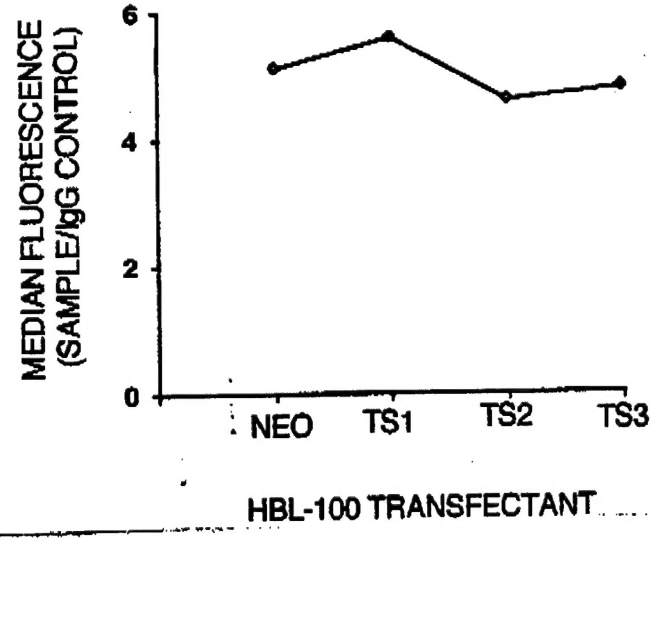
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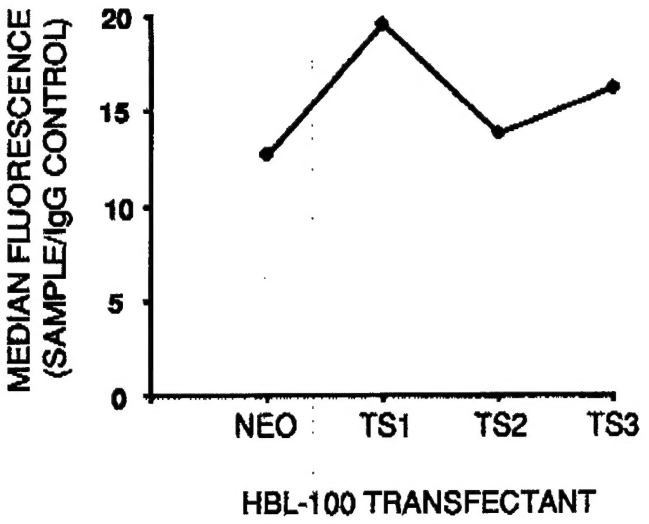
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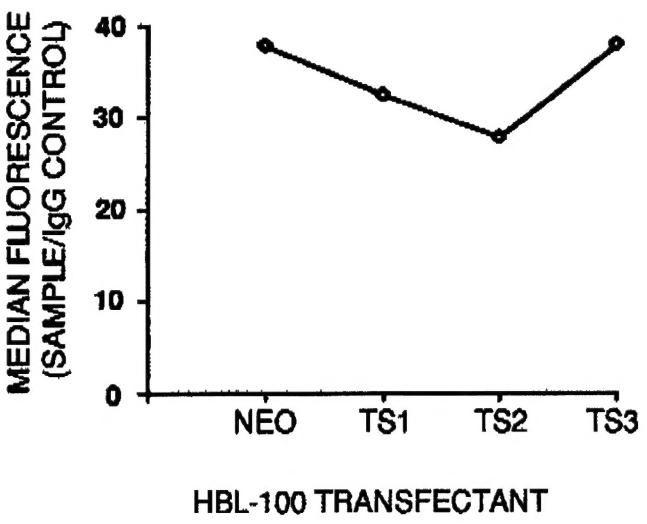
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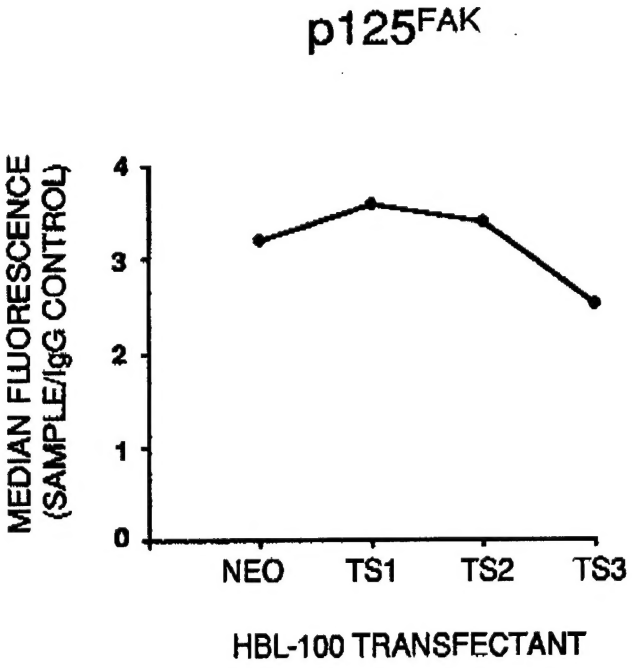
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TS3
HBL-100 TRANSFECTANT

level of overexpression does not exceed the HER-2 level found in the SK-BR-3 cell line which is a natural, non-engineered, overexpressor of HER-2/*neu*. These transfection studies reveal that the number of receptors/cell for some RTK I members are altered by changes in HER-2/*neu* expression. Specifically, HER-1 (EGFR) expression is decreased and HER-3 expression is increased in human breast epithelial cells engineered to overexpress HER-2, while human ovarian epithelial cells show the opposite pattern . The number of HER-4 receptors/cell is considerably lower than those of the other RTKI receptors). This result is not due to limitations in the sensitivity of the assay which can detect as little as 40 molecules/cell, but rather to the known low expression levels of HER-4 in breast cells.

Table 1 Type I receptor quantitation

CELL LINE	HER-1 Receptors/cell	HER-2 Receptors/cell	HER-3 Receptors/cell	HER-4 Receptors/cell
BREAST				
HMEC NEO	1140000	5260 ± 1570	283 ± 54	141 ± 103
HMEC H2	68100	210000	616	71
HBL-100 NEO	36000 ± 207	4450 ± 125	354 ± 119	BDL
HBL-100 H2	29000 ± 265	455000 ± 165	314 ± 105	BDL
MCF-7 PAR	4977 ± 193	15100 ± 73	24600 ± 797	360 ± 15
MCF-7 H2	BDL	1398945 ± 4394	150000 ± 249	296 ± 32
MDA-MB-231 NEO	201000 ± 1125	8090 ± 909	1410 ± 135	BDL
MDA-MB-231 H2	76000 ± 995	115000 ± 126	655 ± 63	BDL
MDA-MB-435 NEO	BDL	925 ± 14	7568 ± 25	BDL
MDA-MB-435 H2	BDL	28100 ± 371	11500 ± 392	BDL
SK-BR-3	40200 ± 157	1957800 ± 1860	12700 ± 135	201 ± 68
OVARIAN				
2008 PAR	109000 ± 150	2460 ± 337	1020 ± 23	300 ± 95
2008 H2	202000 ± 36	104000 ± 339	769 ± 63	115 ± 16
C13 NEO	52500 ± 51	1260 ± 26	654 ± 34	195 ± 48
C13 H2	85100 ± 102	9100 ± 138	653 ± 30	175 ± 52
CaOv3 NEO	746 ± 58	5551 ± 232	5056 ± 285	68 ± 4
CaOv3 H2	BDL	104567 ± 9999	4863 ± 1500	115± 3
DETECTION LIMIT	606	425	59	40

Receptor levels for all four members of the RTKI family in all the cell lines used were quantitated using a modified ELISA technique. Results reflect three independent quantitations, each performed in triplicate and represent the average number of receptors per cell, plus or minus the standard deviation
BDL: BELOW DETECTION LIMIT

Binding of heregulin to two different members of the RTK I family, specifically HER-3[20], and HER-4[21], as well as the documented interactions between these receptors and EGFR (HER-1)[5,24,25,28,46,53-55], suggests that complex combinations of receptor-ligand interactions are occurring in human cells expressing these receptors. Additionally, a recent report on the binding of EGF to HER-2/HER-3 heterodimers[55], indicates that competition between HRG and EGF for binding to this receptor combination might occur *in vivo.* The current results indicate that expression of all four RTK I receptors varies significantly from cell to cell and that HER-2 receptor number can significantly effect expression of the other RTK I members. The results also indicate that biological responses to heregulin both *in vitro* and *in vivo* cell are directly correlated with HER-2/*neu* expression levels. This confirms results from other investigators indicating that heregulin signaling through HER-3 and HER-4 may be dependent on HER-2/*neu* expression levels in breast[46,47] as well as prostate cells[56,57]. Results from the current studies however do not support a significant role for HER-4 as an important molecule in the growth stimulatory HRG-induced effects in human breast and ovarian epithelial cells.

In addition to the above work on agonists in vitro, we have expanded our work on the interactions between antagonists and chemotherapeutic reagents in vitro as part of this Specific Aim.

We have previously reported a synergistic interaction between the anti-HER-2/*neu* antibody 4D5 and CDDP resulting in a two log increase in anti-tumor efficacy in treatment of HER-2/*neu* overexpressing human breast and ovarian carcinoma cells and xenografts (Pietras, *et al.*, 1994), and other studies confirm this interpretation (Hancock, *et al.*, 1991; Arteaga, *et al.*, 1994). Subsequent to this report, the anti-HER-2/*neu* 4D5 antibody was humanized so that it would be suitable for repeated dosing in human subjects without the induction of a human anti-mouse antibodies which have proven to be a limitation for the therapeutic use of other murine monoclonal antibodies (Carter, *et al.*, 1992). To extend the observations made with murine monoclonal antibody 4D5 in combination with CDDP and to conduct a comprehensive survey of rhuMAb HER2 used in combination with other classes of cytotoxic chemotherapeutic drugs available for clinical use, rhuMAb HER2 was analyzed in combination with cytotoxic chemotherapeutic drugs representing seven different drug classes. The drugs studied include an anthracycline antibiotic, doxorubicin (DOX), a taxane, paclitaxel (TAX), the topoisomerase II inhibitor etoposide (VP-16), a platinum analog cisplatin (CDDP), a vinca alkaloid vinblastine (VBL), alkylating agents, thiotepa (TSPA) for *in vitro* experiments and cyclophosphamide (CPA) for *in vivo* experiments, and the antimetabolites methotrexate (MTX) and 5-fluorouracil (5-FU).

In this analysis, dose response curves were constructed for each drug alone, rhuMAb HER2 alone, and the combination at fixed molar ratios. In order to insure the accuracy and reproducibility of the *in vitro* experiments, seeding density of the target SK-BR-3 cells in each plate were carefully controlled such that absorbency values for the control wells were not statistically significantly different from plate to plate, allowing for more precise comparisons between repeated experiments. A representative experiment of the multiple drug effect analyses performed for all chemotherapeutic agents in combination with rhuMAb HER2 is shown using the alkylating agent thiotepa (Figure 1, A and B, Tables 1 and 2). In this analysis Fa and Fu are the fractions of SK-BR-3 cells affected or unaffected, respectively, by the dose (D) of either agent (drug or antibody). Dm is the dose required to produce the median effect (analogous to the IC_{50}), and m is the Hill coefficient used to determine whether or not the dose effect relationships follow sigmoidal dose-response curves. The linear regression correlation coefficients, (r-values) of the median effect plot demonstrate the validity of this methodology (Table 2). CI values for the combination of TSPA and rhuMAb HER2 are significantly less than 1.0 across all combined doses tested indicating a synergistic interaction (Fig. 1B). A summary of the data from the same analysis applied to each of the drugs tested demonstrates that CDDP, TSPA, and VP-16 exhibit synergistic therapeutic interactions ($CI<1$; $P<0.05$) with rhuMAb HER2 in SK-BR-3 human breast carcinoma cells across a wide range (~0.2 - 0.8) of Fa values (Table 3). Additive interactions ($CI=1$) were observed for TAX, DOX, MTX, and VBL in combination with rhuMAb HER2, while only one drug, 5-FU, was found to exhibit an antagonistic ($CI>1$; $p<0.05$) interaction (Table 3).

Table 1. Inhibition of SK-BR-3 cell proliferation by TSPA, and rhuMAb HER2, alone and in combination.

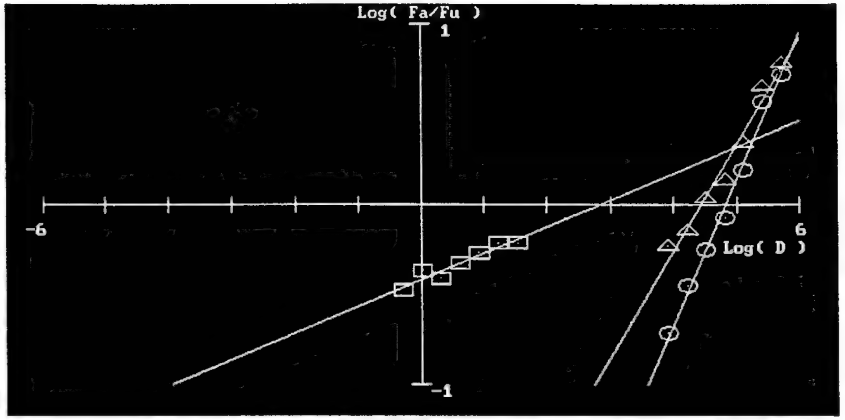
Fractional inhibition (Fa) of:

TSPA (uM)	0	8.3	16.5	33.0	66.1	132.1	264.3	528.5
rhuMAb HER2 (nM)								
0	0	0.16	0.26	0.36	0.46	0.61	0.79	0.84
0.5	0.25	0.37						
1.1	0.3		0.42					
2.1	0.28			0.52				
4.25	0.32				0.58			
8.5	0.35					0.69		
17	0.38						0.82	
34	0.38							0.86

SK-BR-3 cells ($5X10^3$/well) were incubated in triplicate wells as described in the methods section.
The incubation period was 72h.

Table 2. Calculated values for the Combination Index as a function of fractional inhibition of SK-BR-3 cell proliferation by a mixture of TSPA and rhuMAb HER2.

	Combination Index Values at:							
Parameters:								
Drug	ED30	ED40	ED50	ED60	ED70	Dm	m	r
TSPA						66.2uM	0.81	0.99
rhuMAb HER2						675.0nM	0.15	0.96
TSPA+rhuMAb HER2	0.52	0.37	0.41	0.49	0.60	27.1uM	0.59	0.99
Diagnosis of combined effect	Synergy	Synergy	Synergy	Synergy	Synergy			



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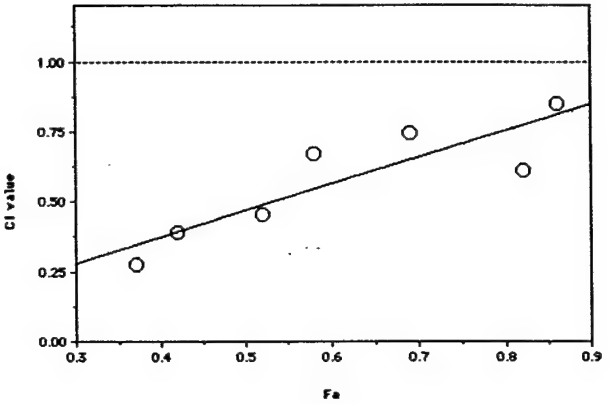

B

Figure Legend

1 (A) Multiple drug effect plot of TSPA, rhuMAb HER2 and the combination. Fa = the fraction of SK-BR-3 cells affected by the drugs, Fu = the fraction of cells unaffected. D = drug dose. (B) Combination Index values for TSPA in combination with rhuMAb HER2 at multiple effect levels. CI values < 1 indicate synergy.

Table 3. Mean combination index values for chemotherapeutic drug/rhuMAb HER2 combinations *in vitro.*

Drug	rhuMAb HER2/Drug Molar Ratio	Drug Dose Range (uM)	Combination Index (Mean ± SEM)	P Value	Interaction
TSPA	6.4×10^{-5}	$8.25 - 1.06 \times 10^{3}$	0.67 ± 0.12	0.0008	Synergy
CDDP	4.0×10^{-4}	$6.5 \times 10^{-1} - 1.7 \times 10^{2}$	0.56 ± 0.15	0.001	Synergy
VP-16	9.9×10^{-4}	$2.6 \times 10^{-1} - 6.8 \times 10^{1}$	0.54 ± 0.15	0.0003	Synergy
DOX	9.8×10^{-3}	$2.7 \times 10^{-2} - 6.9$	1.16 ± 0.18	0.13	Addition
TAX	1.4×10^{-1}	$1.8 \times 10^{-3} - 5.0 \times 10^{-1}$	0.91 ± 0.23	0.21	Addition
MTX	3.3×10^{-1}	$8.0 \times 10^{-4} - 2.0 \times 10^{-1}$	1.36 ± 0.17	0.21	Addition
VBL	1.7	$1.6 \times 10^{-4} - 3.9 \times 10^{-2}$	1.09 ± 0.19	0.26	Addition
5-FU	8.8×10^{-5}	$3.0 - 7.65 \times 10^{2}$	2.87 ± 0.51	0.0001	Antagonism

Specific Aim III - To assess the biologic effects of ligand and antibody, alone and in combination, on HER-2/*neui* expressing human brast cancer cells in vivo.

During the previous progress report record, we completed studies on the in vivo effects of heregulin on tumorigenicity of HER-2/*neu* overexpressing cells. During the most recent funding period we have evaluated the in vivo effects of chemotherapeutic drugs incombination with the anti-HER-2/*neu* antibody, 4D5 or rhu Mab HER-2. In addition we have expanded the work to evaluate the effects of the combination of anti-HER-2 antibodies with radiation therapy.

To further evaluate the potential therapeutic effects of rhuMab HER2/chemotherapy combinations and to extend our observations beyond a single cell line and preclinical model, a series of *in vivo* studies of chemotherapy with rhuMAb HER2 against human breast cancer xenografts in athymic mice were performed. All of the doses, routes of administration, and dose intervals for the various cytotoxic drugs and rhuMAb HER2 were based on independent dose finding experiments for this specific strain, age, weight, and sex of athymic mouse. The cytotoxic drug doses used were at or near the maximum tolerated doses previously reported in the literature.

For the alkylating agent cyclophosphamide CPA, combination with rhuMAb HER2 resulted in a significant reduction ($P<0.05$) in day 21 xenograft volume compared to either agent alone (Figure 2A). The combination of the anthracycline antibiotic DOX plus rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to either single agent alone (Figure 2B). The combination of the taxane compound TAX plus rhuMAb HER2, which demonstrated an additive interaction *in vitro*, resulted in a significant reduction in day 20 xenograft volume compared to treatment with TAX alone (Figure 2C). However, the difference between rhuMAb HER2 alone and rhuMAb HER2 plus TAX did not reach statistical significance. This is likely due to the relatively small sample size in each group and the fact that the dose of rhuMAb HER2 in this particular analysis (10mg/kg IP twice weekly) yielded a marked reduction in xenograft growth even when used as a single agent.

The following four drug/rhuMAb HER2 combinations were studied in a single large experiment. For this experiment, a "rational dose" (RD) of rhuMAb HER2 was chosen as new information became available based on comparative pharmacokinetic studies from both humans and athymic mice. RD is the dose of a given drug which can reproduce a serum level in nude mice similar to that observed in human subjects. The RD for rhuMAb HER2 resulted in a lower cumulative rhuMAb HER2 dose (16mg/kg vs. 30-50mg/kg) during the 21 day observation period for this experiment compared to the three*in vivo* studies reported above. With this approach, a

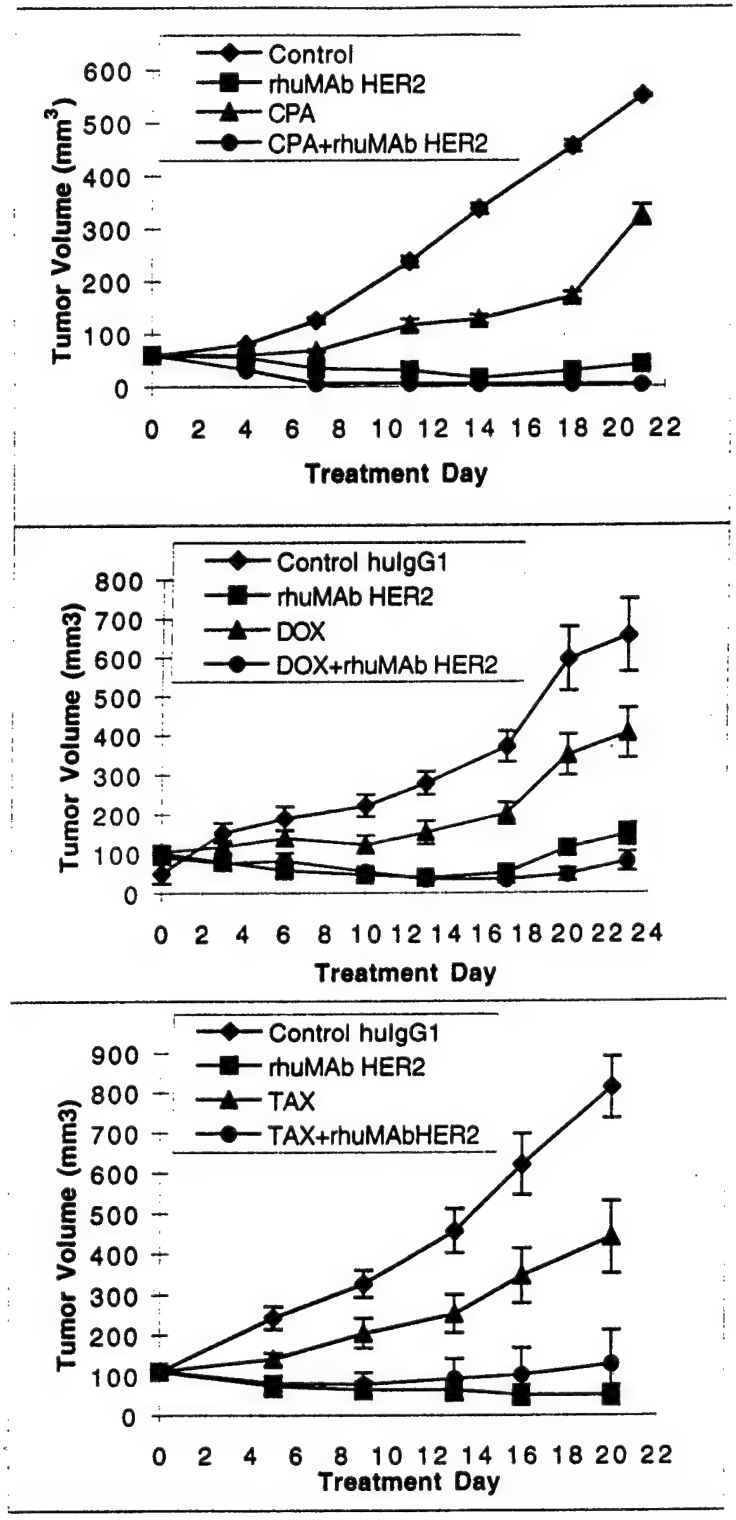

Figure Legend

2 Combination treatment of MCF7/HER-2 breast carcinoma xenografts in athymic mice with rhuMAb HER2 plus CPA (A), DOX (B), and TAX (C).

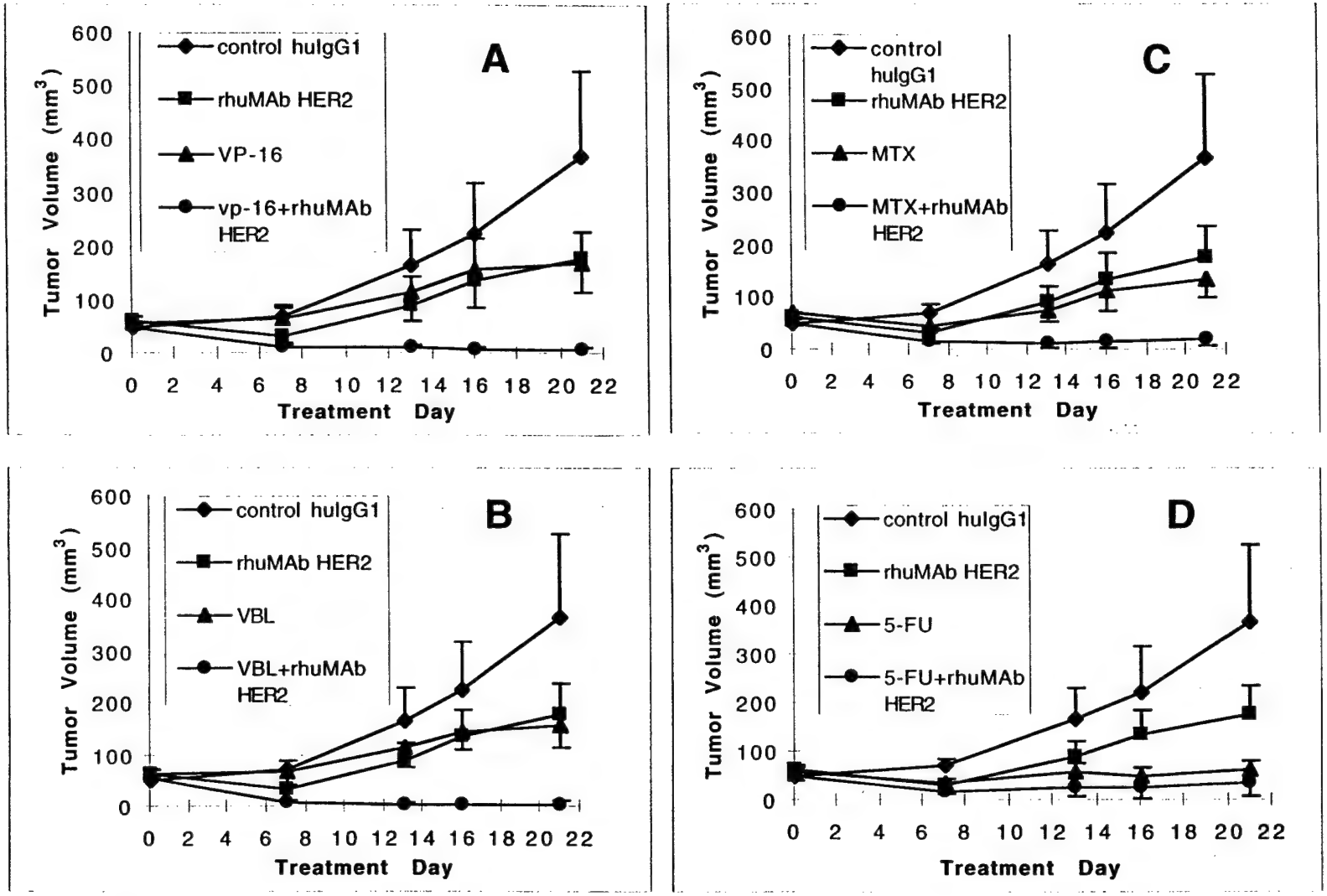

Figure Legend

3. Treatment of MCF7/HER2 xenografts with rhuMAb HER2 in combination with VP-16 (A), VBL (B), MTX (C), and 5-FU (D).

significant reduction in day 21 xenograft volume was observed for the topoisomerase II inhibitor VP-16 when used in combination with rhuMAb HER2 compared to either agent alone (Figure 3A). The combination of the microtubule inhibitor VBL with rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to treatment with VBL alone or single agent rhuMAb HER2 (Figure 3B). For the antimetabolite class of cytotoxic chemotherapeutics, two drugs with clinical activity against breast cancer were chosen for combination studies. Treatment with MTX, which targets dihydrofolate reductase, in combination with rhuMAb HER2 resulted in a significant reduction in day 21 MCF7/HER-2 xenograft volume when compared to either MTX alone or rhuMAb HER2 alone (Figure 3C). However, the antimetabolite drug 5-FU, which targets thymidylate synthetase, and which was found to be antagonistic when combined with rhuMAb HER2 *in vitro*, did not yield a significant reduction in xenograft volume when compared to 5-FU alone *in vivo* (Figure 3D). Although the combination of rhuMAb HER2 plus 5-FU was superior to rhuMAb HER2 alone in this experiment ($P<0.05$), the 5-FU dose used had sufficient efficacy as a single agent such that it was not possible to resolve potential differences between 5-FU alone and the combination with the sample sizes chosen (10 mice/group).

As stated above the work on synergy between radiation therapy and anti-HER-2 antibodies has also proven quite interesting and has been conducted under this specific aim. We have directly compared the radiation sensitivities of parental breast cancer cells with normal expression levels of HER-2 with that of matched daughter cells containing HER-2 overexpression. Survival curves were obtained after treatment of cells with graded doses of ionizing radiation at a dose rate of 1 Gy / min with doses of 0, 1, 2, 4 and 5 Gy. After the radiation treatment, cells were placed into 35-mm dishes and cultured for 14 days, with the surviving fractions quantitated at day 14 (*20*). Irradiation of MCF-7/HER-2 cells *in vitro*, resulted in a D_{10} (the dose required to reduce cell survival to 10%) which was increased by approximately 25% relative to MCF-7/ control cells. These analyses suggest a potential biological role of the HER-2 oncogene in resistance to radiation treatment. Alternatively, this apparent difference in sensitivity could be the result of a differential growth rate, since HER-2-overexpressing cells have been found to exhibit more rapid regrowth than control cells following the initial response to chemotherapy.

To evaluate the effect of radiation combined with antibodies to HER-2, we conducted studies using the transfected MCF-7 cells as well as the naturally HER-2 overexpressing SKBR3 breast cancer cells. Cells cultivated either on plastic (MCF-7/HER-2 cells) or in soft agar plates (SKBR3 cells) with or without rhuMAb HER-2 for 12h, were treated with or without radiation prior to incubation for 2 weeks. These data show that anti-HER-2 receptor antibody enhances radiation-induced killing of human breast cancer cells with HER-2 overexpression .

To evaluate the efficacy of radiation therapy with rhuMAb HER-2 on the growth of MCF-7 HER-2 xenografts, cells were innoculated into estrogen-primed female athymic mice for 14 days and allowed to grow to 50-100mm^3 prior to randomization to 4 groups. Treatment groups included control human IgG1 at 30 mg/kg (CON), radiation at 4 Gy with human IgG1 (RT), rhuMAb HER-2 at 30 mg/kg (MAb) or combined radiation / rhuMAb (RT/MAb) therapy (Fig.4). Doses of antibody or IgG1 were administered in divided doses on days 1,4 and 7. Those groups treated with radiation received a treatment 4h after administration of antibody or control IgG1 on days 1,4 and 7. Tumor nodules were monitored through day 49. The effect of repeated doses of rhuMAb HER-2 with or without ionizing radiation on tumor volume in the various groups was measured (Figure 5). In mice receiving low doses of radiation with control IgG (RT), mean tumor volumes were not significantly reduced over the 7-week observation period and no tumor remissions were observed when compared to controls (CON). Tumors treated with rhuMAb HER-2 alone (MAb) also failed to show significant growth reduction as compared to controls and again no tumor remissions were noted. In contrast combined radiation-antibody therapy produced marked reduction in tumor volumes over the 7-week treatment period when compared to control or either treatment alone ($P< 0.001$). Moreover all animals that received both rhuMAb HER-2 and radiation (RT/MAb) had complete tumor remissions. These data show superior efficacy of radiation when given with rhuMAb HER-2 and demonstrate a clear therapeutic advantage with this treatment regimen.

After demonstrating a therapeutic advantage for the combination of antibody and radiation in HER 2-overexpressing cells, studies were designed to evaluate the possible mechanism(s) for this phenomenon. Studies have shown that DNA repair plays an important role in the recovery of cells from the toxicity of ionizing radiation. Prior work has also shown that inhibition of DNA repair by anti-HER-2 receptor antibodies are important in antibody-enhanced cytotoxicity of cisplatin in HER-2-overexpressing breast and ovarian cancer cells. To evaluate whether similar alterations in DNA repair may be a potential explanation for the enhanced effects of antireceptor antibody and radiation, we measured DNA repair induced by radiation in SKBR3 and MCF-7/HER-2 cells using autoradiographic localization of [^{3}H]thymidine over cell nuclei to provide a quantitative measure of this phenomenon. As expected radiation exposure induces enhanced unscheduled DNA synthesis in SKBR3 cells. Exposure to rhuMAb HER-2 alone has no such effects on these cells. The radiation-induced effect, however, was blocked by pre-treatment of the cells with antireceptor antibody. To determine whether this phenomenon was restricted to a specific cell line, and to study its association with HER-2 overexpression, we performed similar studies in MCF-7 and MCF-7/HER-2 cells. These two cell lines are identical to one another except for the presence of HER-2 overexpression in the MCF-7/HER-2 cells. Radiation elicits a marked increase in DNA repair in the PAR, CON and HER-2 cells. However, this radiation-induced effect is blocked by rhuMAb HER-2 in the MCF-7 HER-2 cells specifically and does not occur in control cells. These data confirm that rhuMAb HER-2 interferes with DNA repair only in those cells overexpressing the HER-2 receptor . Using an alternative measure of DNA repair, we observe the same phenomenon. with a transfected cmu drive ß-galactosidase reporter plasmid. At 24h post transfection, the extent of repair was assayed by measuring reporter DNA expression in MCF-7/ HER-2 cells that were either incubated with rhuMAb HER-2 or control media after transfection (0 hours). The transfected cells were then stained with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, a substrate for ß-galactosidase, to distinguish ß-galactosidase-positive and -negative cells. In the presence of substrate, cells expressing the reporter bacterial ß-galactosidase protein appear blue and the percentage of stained cells can be quantitated. These data demonstrate that antibody treatment elicits blockade of repair of the radiation-damaged reporter DNA, again demonstrating a therapeutic advantage seen in cells overexpressing HER-2 after treatment with a combination antibodies to the HER-2 receptor and radiation.

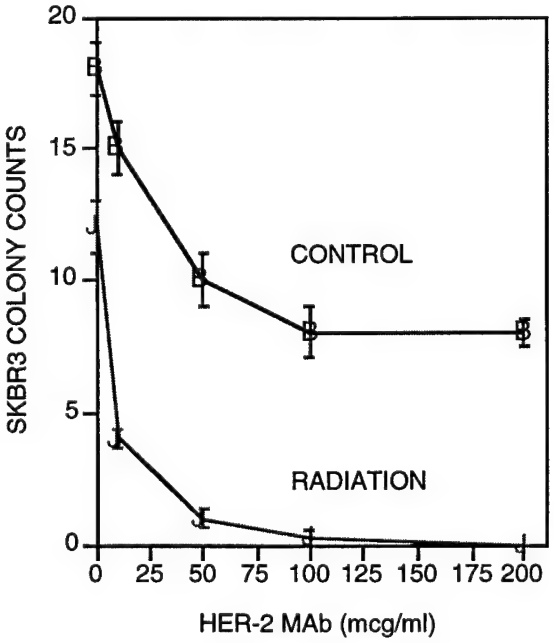

Figure 4

Human breast cancer cells with overexpression of HER-2 growth factor receptor show radiation resistance *that can be reversed by treatment with antibody to HER-2 receptors (A).* Survival curves were obtained for cells after treatment with graded doses of ionizing radiation at a dose rate of 1 Gy / min with doses of 0, 1, 2, 4, and 5 Gy. The treatment groups included MCF-7 control breast cancer cells with low-expression of HER-2 (squares), HER-2-overexpressing MCF-7 breast cancer cells (circles) and HER-2-overexpressing MCF-7 cells treated with recombinant humanized monoclonal antibody to HER-2 receptor at 200 μg per ml beginning 2h prior to radiation (triangles). After radiation treatment, cells were divided into 35 mm dishes and cultured over 14 days. Curves are plotted as the mean $\pm$ SE from three experiments. Effects of HER-2 gene overexpression on increased cell survival are significantly different from control ($P<0.05$), and treatment with antibody to HER-2 receptor is associated with a reversal of this radioresistance ($P<0.05$).

(B) Human breast cancer cells with natural overexpression of HER-2 receptor, SKBR3, were treated in vitro with rhuMAb HER-2 at 0-200 μg/ml, with or without adjuvant radiation treatment at 2 Gy. To study the effect of rhuMAb HER-2 on ancorage-independent growth, cells were plated at a density of 2.5×10^4 cells / 6-cm dish in triplicate. The dishes consist of a 0.4% (w/v) agar bottom layer, and a 0.2% (w/v) agar top layer. Cells were plated in between the latter two layers with rhuMAb HER-2 or control solution. Plates were irradiated at 2 Gy or maintained as non-irradiated controls, then incubated at 37 C , 5% CO2 for 3 weeks, at which time colonies were counted.

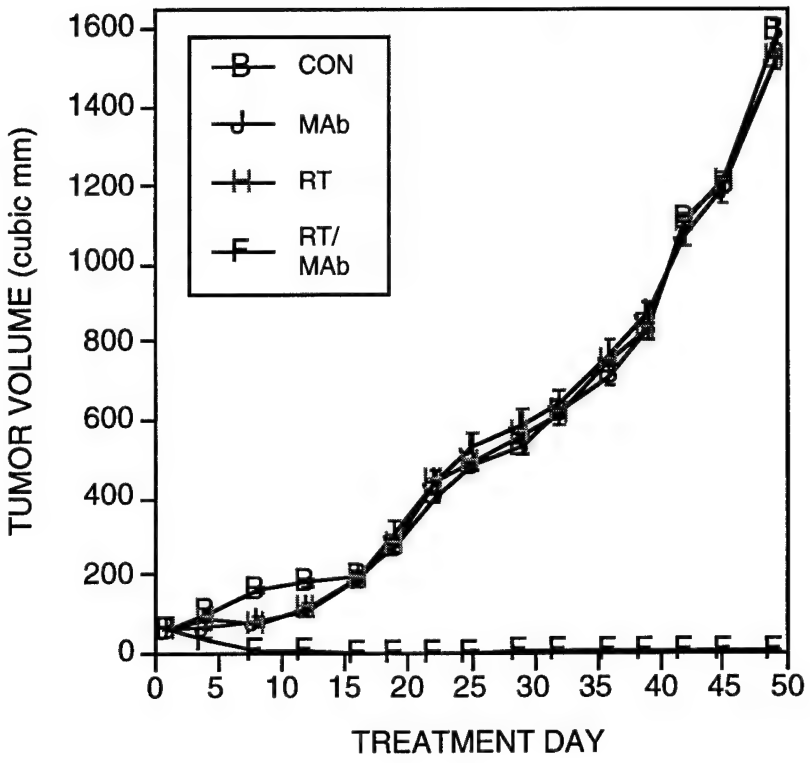

Figure 5.

Combination treatment with antibody to HER-2 growth factor receptor and radiation promotes remission of human breast cancer xenografts in nude mice. MCF-7 / HER-2 cells were injected subcutaneously at 5 x 10^7 cells per nude mouse. After 14 days, mice were randomized on day 0 to groups of 6 animals on the basis of body weight and tumor nodule size. Treatment groups included human IgG1 control at 30 mg/kg (CON-square), radiation at 4 Gy with human IgG1 (RT-triangle), rhuMAb HER-2 at 30 mg/kg (MAb-circle) or combined radiation / rhuMAb (RT/MAb-diamond) therapy. Doses of antibody or IgG1 indicated above were administered in divided doses on days 1,4 and 7. Those groups treated with radiation received a treatment 4h after administration of antibody or IgG1 on days 1,4 and 7 only. Tumor nodules were monitored to day 49. On postmortem examination, no residual tumor cells were found by light microscopy in the RT/MAb treatment group, but cancer cells were found in the subcutaneous nodules of mice from all other treatment groups.

Specific Aim IV - To assess the biologic effects of an additional mutation of the p53 gene in combination with HER-2/*neu* overexpression in human breast cells both in vitro and in vivo.

As presented in last year's progress report we have had difficulties achieving this specific aim due to problems with stably expressingar mutant p53 gene in the human breast cancer cells we have engineered in specific aim 1. To date, we have been unable to obtain stable transfectants expressing both a mutant p53 and overexpressing HER-2/*neu* using our vector systems and gene constructs. As a result we have changed the focus of the specific aim to study the interaction of the HER-2 alteration and the estrogen receptor (ER). This continues to be in keeping with the effects of additional changes in a second gene (ER) albeit not a structural mutation in the gene. As stated above, however, we have identified a potentially important interaction between HER-2/*neu* and the estrogen receptor and are pursuing it as part of this specific aim.

Estrogen receptor functions as a nuclear transcription factor. On binding estradiol, ER interacts with estrogen-responsive elements (ERE) in the nucleus to modulate transcription of target genes such as the progesterone receptor. Activation of ER ultimately leads to its down-regulation in those cells expressing it. Phosphorylation of ER occurs early in its activation and is now known to be associated with functional changes induced by the interaction of ER with ERE in the nucleus. Blockade of this pathway through interfering with the binding of estrogen to receptors is the basis of the current major hormone treatment modality, tamoxifen. Tamoxifen is a partial agonist and is believed to work by competitively binding to ER, thereby limiting the proliferative effects of estrogen. Resistance to tamoxifen, however, has been reported in women whose breast cancers overexpress HER-2/*neu*. On stimulation by ligand the intrinsic tyrosine kinase activity of HER-2/*neu* is activated and appears to promote signal transduction to the nucleus along specific phosphorylation cascades. Recent data from our laboratory indicates that phosphorylation of ER is also linked to the HER-2/heregulin growth factor pathways. Stimulation of the HER-2/*neu* receptor by heregulin or overexpression of this receptor can induce many of the same biochemical and functional changes in the ER even in the absence of estrogen. These changes include phosphorylation of ER, increase in DNA binding, activation of the ERE, transcription of the progesterone receptor and ultimate down-regulation of the ER. These data may in part explain the association between HER-2/*neu* overexpression and ER-negativity, as well as the estrogen independence and tamoxifen resistance seen in clinical practice in those tumors with the HER-2 alteration.

To further evaluate the relationship of HER-2/*neu* overexpression to clinical antiestrogen resistance, we have employed estrogen-responsive, human breast cancer cells with defined levels of ER which have been transfected with the HER-2/*neu* gene. These transfectants are identical to the parental, non-HER-2/*neu* transfected cells with the exception of HER-2/*neu* overexpression. These parental and transfected cells were then evaluated for their response to antiestrogens as well as the development of an estrogen-independent phenotype *in vivo*. Finally, therapeutic approaches directed at HER-2/*neu* overexpression were undertaken and effects on the hormone-independent, tamoxifen-resistant phenotype of these cells were measured *in vivo*. The results of these studies indicate an important and potentially clinically exploitable link between the HER-2/*neu* and ER pathways in human breast cancer.

As mentioned above, overexpression of HER-2/*neu* receptor occurs in 25-30% of human breast cancers and is associated with an ER-negative phenotype in 65% of those cases as well as a poor clinical response to tamoxifen therapy in the 35% which are ER-positive and which also overexpress HER-2/*neu*.

In previous *in vitro* studies from our laboratory, rhuMAb HER-2 was found to restore the growth inhibitory response to tamoxifen in HER-2/*neu* overexpressing MCF-7 breast cancer cells that are otherwise resistant to tamoxifen. To determine whether this return to tamoxifen sensitivity would occur *in vivo*, we conducted a series of experiments with MCF-7 human breast cancer xenografts in nude mice. MCF-7 cells with or without overexpression of HER-2/*neu*

receptor were grown as subcutaneous xenografts as previously described. As expected, growth of MCF-7 control cells is completely suppressed by tamoxifen, a classical antiestrogen, as well as by ICI 182,780 a pure antiestrogen (Figure 6a). In contrast, MCF-7 cells with HER-2/*neu* receptor overexpression are completely insensitive to tamoxifen and only partially sensitive to ICI 182,780 (Figure 6b). Treatment of HER-2/*neu*-overexpressing MCF-7 xenografts with the 4D5 monoclonal antibody to HER-2/*neu* receptor in combination with tamoxifen restores *in vivo* sensitivity to tamoxifen therapy to levels found in MCF-7 control cells (Figure 7). These *in vivo* studies demonstrate a clear growth-inhibitory therapeutic benefit of the combination of rhuMAb HER-2 with tamoxifen in HER-2/*neu*-overexpressing human breast cancer xenografts.

To better understand the possible molecular basis for the *in vivo* observations, we conducted a series of studies to evaluate the interaction between ER and a nuclear ERE in the presence and absence of the antibody. Since ER-ERE binding is prerequisite for activation of ER-induced transcription, regulation of ERE by antiestrogens was evaluated in MCF-7 cells transiently transfected with a plasmid containing an ERE upstream of a chloramphenicol acetyltransferase (CAT) gene. This construct is responsive to estradiol-17ß in MCF-7 control cells and the effect is abolished by preincubation of the cells with either tamoxifen or the pure antiestrogen, ICI 182,780. The latter of these two antiestrogens is believed to act at the level of the ER-ERE interaction. The ERE-CAT construct was also transfected into MCF-7 cells containing overexpression of the HER-2/*neu* receptor. Even in the absence of estrogen, MCF-7 HER-2 cells contain enhanced ERE-CAT gene activity when compared to MCF-7 control cells; however, treatment with estradiol will elicit an incremental increase in CAT expression (Fig. 8). Tamoxifen is ineffective in disrupting this activity in HER-2/*neu*- overexpressing cells; however, the effect can be significantly reduced by incubation with the pure antiestrogen, ICI 182,780 (Fig.8) ($P<0.01$). Treatment with the combination of tamoxifen and the anti-HER-2 monoclonal antibody in these cells restores the expected inhibitory effect of tamoxifen (Fig. 9). These data demonstrate that overexpression of the HER-2/*neu* receptor contributes to regulation of ER transcriptional activity and may distort normal regulatory pathways. The data also demonstrate that interaction between the HER-2/*neu* receptor and antireceptor antibody enhances the therapeutic efficacy of antiestrogens in breast cancer cells containing HER-2/*neu* overexpression.

To evaluate potential molecular sites for cross-communication between HER-2/*neu* signaling and ER signaling, tyrosine phosphorylation of the ER as well as effects of treatment with estrogen, antiestrogen and anti-HER-2/*neu* receptor antibody were investigated. Earlier studies suggested that tyrosine residues in ER may be affected by HER-2/heregulin signaling pathways. Control MCF-7 cells which do not overexpress HER-2/*neu* were treated with control solution or 2 nM estradiol-17ß for 5-15 minutes, then assessed for tyrosine phosphorylation of ER by established methods. Estradiol-17ß, the natural agonist for ER, elicits a rapid tyrosine phosphorylation of its receptor in MCF-7 control cells, with substantial effects evident by 10-15 minutes (Figures 10 a,b)). In contrast, tamoxifen, a synthetic partial agonist for ER, or the combination of tamoxifen with estradiol elicits reduced stimulation of tyrosine phosphorylation of ER in these same cells at 15 minutes (Fig. 10a). A similar, but more pronounced, suppression of ER tyrosine phosphorylation is also found with the pure antiestrogen antagonist, ICI 182,780, either alone or in combination with estradiol (Fig. 10b). In MCF-7/HER-2 cells which contain HER-2/*neu* overexpression, the basal level of tyrosine phosphorylation of ER is increased as compared to MCF-7 control cells, and treatment with estradiol elicits only a moderate degree of additional phosphorylation (Fig. 10c,d). In addition, tamoxifen or tamoxifen plus estradiol enhances, rather than blocks, the basal level of tyrosine phosphorylation of ER (Fig. 10c), whereas ICI 182,780, alone or in combination with estradiol, demonstrates reduced tyrosine phosphorylation of ER. The ICI 182,780 effect is what might be predicted for an estrogen antagonist, but this antagonistic effect is less than that found in MCF-7control cells (Fig. 10d). In MCF-7/HER-2 cells, treatment with the anti-HER-2 monoclonal antibody 4D5 alone has little effect on the tyrosine phosphorylation of ER (Fig. 10e), however, use of anti-HER-2 antibody in combination with tamoxifen and estradiol results in restoration of the expected inhibitory effects of tamoxifen on ER tyrosine phosphorylation (Fig. 10e). These results suggest that the state of tyrosine phosphorylation of ER may be important in regulating its biologic activity.

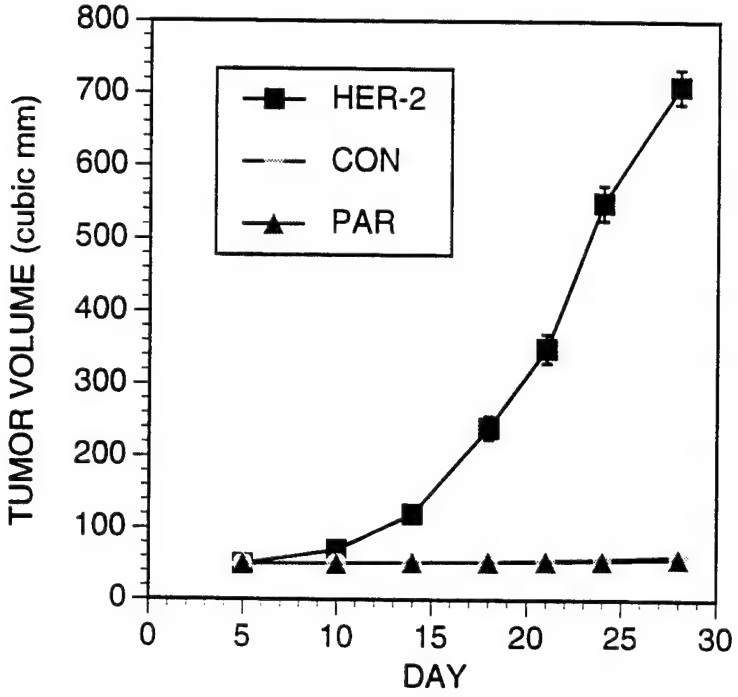

Figure 6 . Overexpression of HER-2 gene in MCF-7 cells elicits resistance to endocrine therapy *in vivo*. **a**, Antiestrogen sensitivity of MCF-7 parental cells without HER-2/*neu* overexpression (control) *in vivo*. MCF-7 cells were inoculated sc in ovariectomized, athymic mice which were primed with estrogen. After 10 days, animals with tumors of comparable size were randomized to treatment with tamoxifen (TAM; 5 mg sustained-release pellet s.c.), ICI 182,780 (ICI; 5 mg in castor oil sc once per week) or control vehicle (CON) for 28 days. Tumor volumes of MCF-7 parental control cells with and without antiestrogen therapy were recorded. No significant difference in cellular accumulation of [^{3}H]-tamoxifen by MCF-7 parent as compared to MCF-7 HER-2 cells was found in three experiments (data not shown).
b, Antiestrogen sensitivity of MCF-7 cells with HER-2/*neu* overexpression (HER-2) *in vivo*. Cells with HER-2/*neu* overexpression were inoculated sc in ovariectomized, athymic mice which were primed with estrogen. After 10 days, animals with tumors of comparable size were randomized to therapy with tamoxifen (TAM), ICI 182,780 (ICI) or control vehicle (CON) as in Fig.1a above.

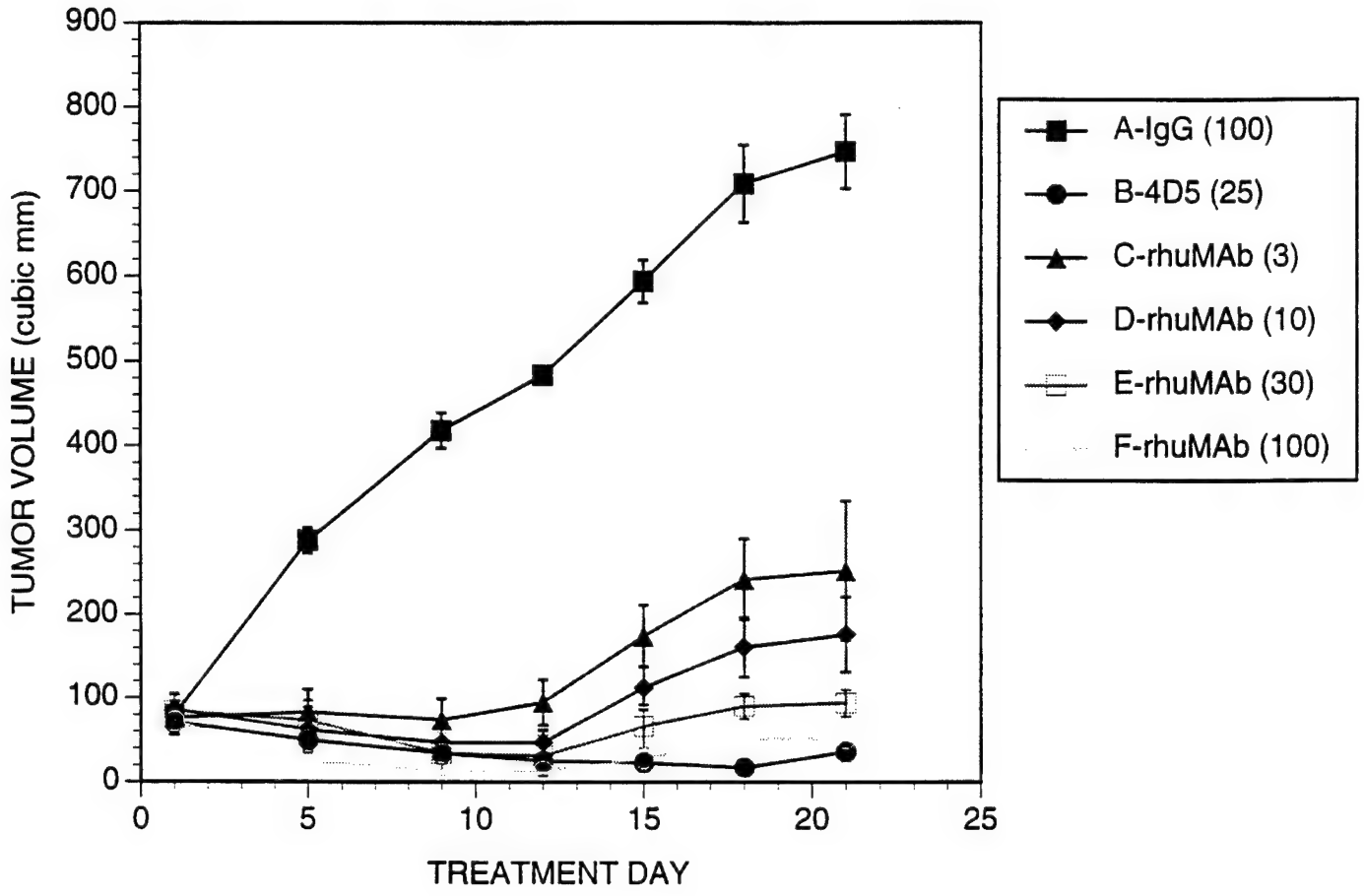

Figure 7. Reversal of tamoxifen resistance in human breast cancer cells with HER-2/*neu* overexpression. The therapeutic benefit of tamoxifen with rhuMAb HER-2 was evaluated using HER-2/*neu*-overexpressing MCF-7 breast cancer cells grown as xenografts. The resulting tumors were treated with control injection (CON), tamoxifen (TAM, 5 mg sustained release pellet sc), monoclonal antibody to HER-2/*neu* receptor (MAb) alone (10 mg/kg every 4 days) or monoclonal antibody to HER-2/*neu* receptor in combination with tamoxifen (TAM/MAb). See text for additional details.

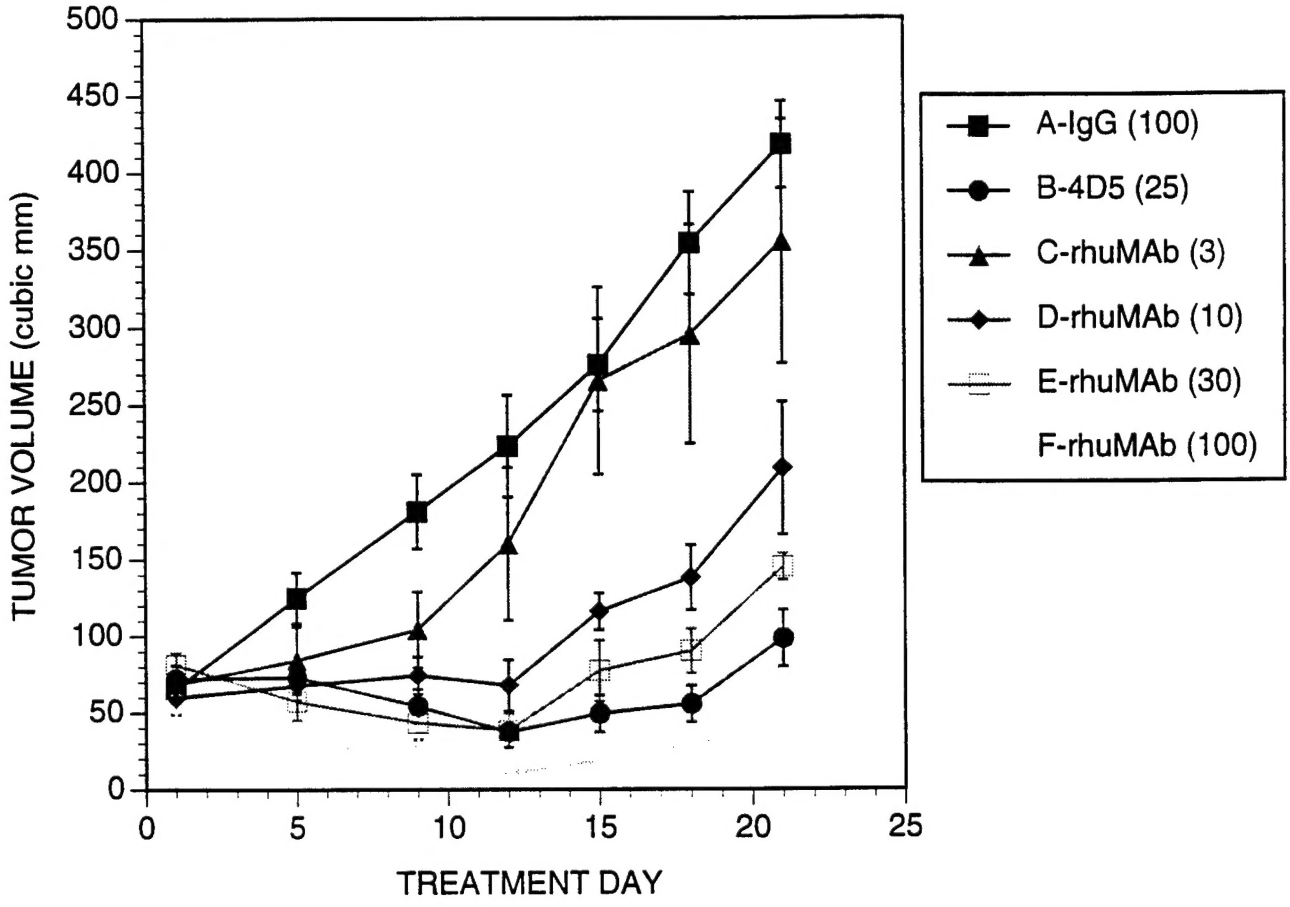

Figure 8. Modulation of the effects of antiestrogens on the interaction between estrogen receptor and estrogen-responsive elements in MCF-7 cells with HER-2/*neu* overexpression. Regulation of estrogen-responsive elements in DNA by antiestrogens was tested in MCF-7HER-2 cells transiently transfected with a reporter plasmid containing an ERE upstream of a chloramphenicol acetyltransferase (CAT) gene. Substituting the basic reporter plasmid pBLCAT2 for pERE-BLCAT offers an additional control for specificity of the DNA-binding site in the regulatory sequence of the reporter gene (ERE). MCF-7 cells were used to establish transient transfection assays that allow the determination of ERE-dependent induction of CAT activity. CAT protein was assessed by established methods (52) after treatment with control (Con), estradiol-17ß at 2 nM (E2), tamoxifen at 1 µM (Tam), tamoxifen with estradiol (Tam/E2), ICI 182,780 at 10 nM (ICI) or ICI 182,780 with estradiol (ICI/E2). See text for additional details.

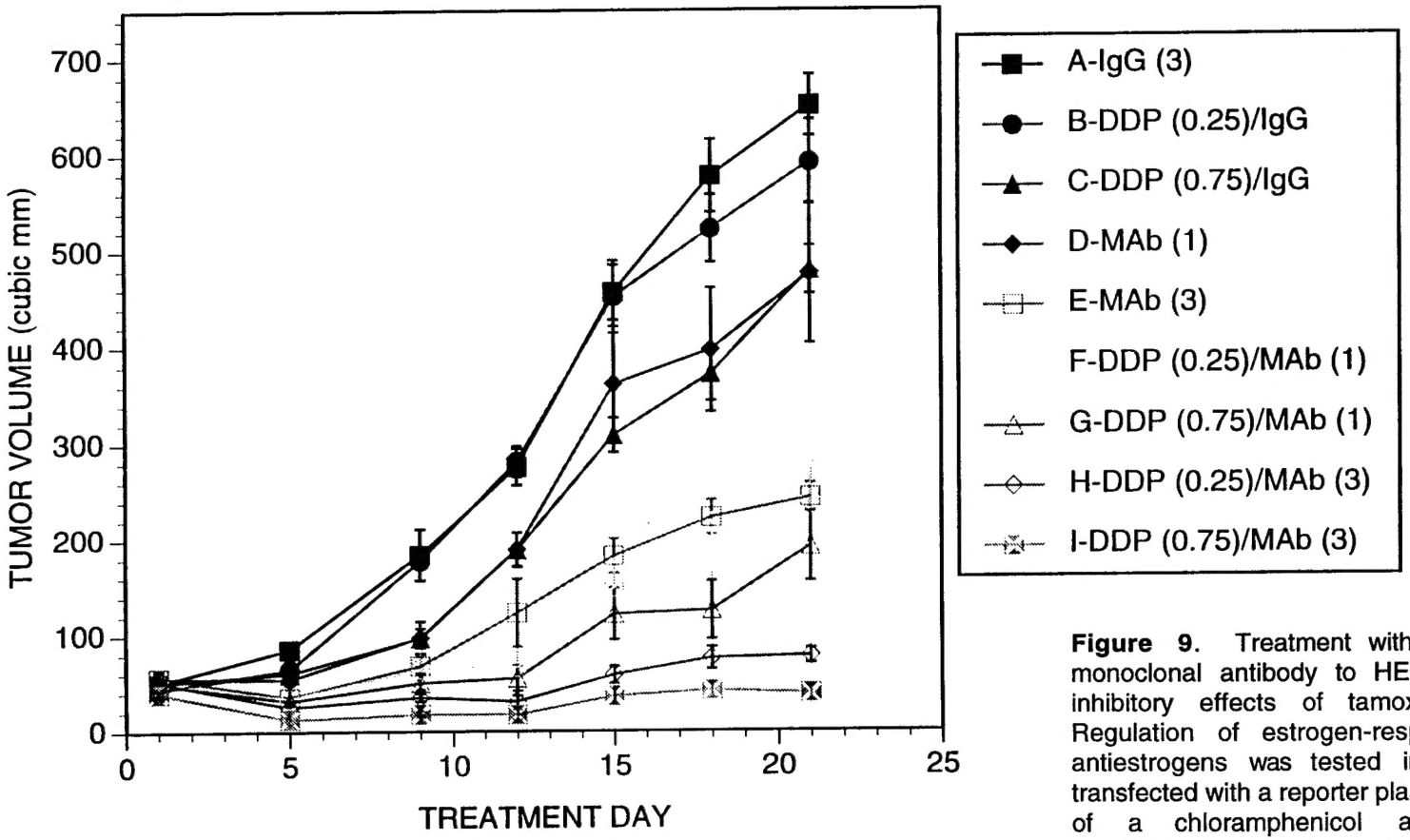

Figure 9. Treatment with tamoxifen in combination with monoclonal antibody to HER-2/*neu* receptor facilitates the inhibitory effects of tamoxifen on ER-ERE interactions. Regulation of estrogen-responsive elements in DNA by antiestrogens was tested in MCF-7HER-2 cells transiently transfected with a reporter plasmid containing an ERE upstream of a chloramphenicol acetyltransferase (CAT) gene. Substituting the basic reporter plasmid pBLCAT2 for pERE-BLCAT offers an additional control for specificity of the DNA-binding site in the regulatory sequence of the reporter gene (ERE). MCF-7 cells were used to establish transient transfection assays that allow the determination of ERE-dependent induction of CAT activity. CAT protein was assessed by established methods (52) after treatment with control solution (Con), monoclonal antibody to HER-2/*neu* receptor at 100 µg/ml (Ab), monoclonal antibody with 2 nM estradiol-17ß (Ab/E2), monoclonal antibody with 1 µM tamoxifen (Ab/Tam), and monoclonal antibody with tamoxifen and estradiol (Ab/Tam/E2). See text for additional details.

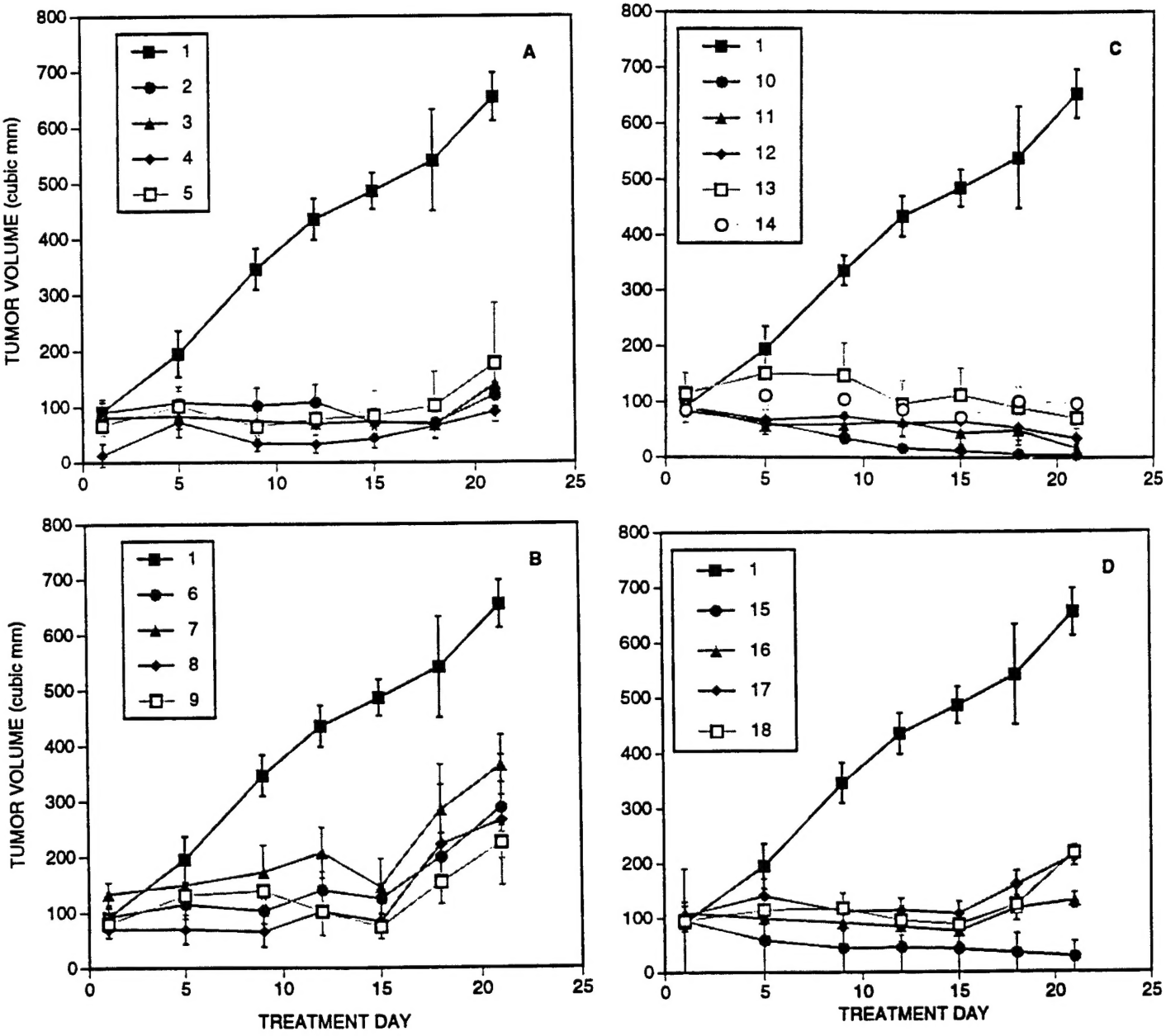

Figure 10. Tyrosine phosphorylation of the estrogen receptor and effects of treatment with estrogen, antiestrogen and anti-HER-2/*neu* receptor antibody. Human breast cancer cells were assessed for the extent of tyrosine phosphorylation of estrogen receptor by immunoprecipitation of cell lysates with antibody to phosphotyrosine and immunoblotting with antibody to estrogen receptor by established methods (15). The estrogen receptor protein occurs at 67 kd. **a**, MCF-7 cells without HER-2/*neu* overexpression (MCF-7/CON) were treated with control solution (Cn), 2 nM estradiol-17ß for 5, 10 or 15 minutes, 1 µM tamoxifen for 15 minutes (TM) or a combination of tamoxifen and estradiol for 15 minutes (TM/E). **b**, MCF-7 cells without HER-2/*neu* overexpression (MCF-7/CON) were treated with control solution (Cn), 2 nM estradiol-17ß for 5, 10 or 15 minutes, 10 nM ICI 182,780 for 15 minutes (ICI) or a combination of ICI 182,780 and estradiol for 15 minutes (ICI/E). **c**, MCF-7 cells with HER-2/*neu* overexpression (MCF-7/HER2) were treated with control solution (Cn), 2 nM estradiol-17ß for 5, 10 or 15 minutes, 1 µM tamoxifen for 15 minutes (TM) or a combination of tamoxifen and estradiol for 15 minutes (TM/E). **d**, MCF-7 cells with HER-2/*neu* overexpression (MCF-7/HER2) were treated with control solution (Cn), 2 nM estradiol-17ß for 5, 10 or 15 minutes, 10 nM ICI 182,780 for 15 minutes (ICI) or a combination of ICI and estradiol for 15 minutes (ICI/E). **e**, MCF-7 cells with HER-2/*neu* overexpression (MCF-7/HER2) were treated with control solution (Cn), 2 nM estradiol-17ß for 15 minutes (E2), 100 µg/ml monoclonal antibody to HER-2/*neu* receptor for 15 minutes (Ab), 1 µM tamoxifen for 15 minutes (TM), tamoxifen with estradiol for 15 minutes (TM/E) or monoclonal antibody with tamoxifen and estradiol for 15 minutes (Ab/TM/E).

CONCLUSION

The data developed during the past 12 months have been extremely exciting and potentially exploitable clinically. All of these data represent an expansion of our knowledge base regarding the role HER-2/*neu* plays in aggressive subtypes of breast cancer including;

A) Overexpression of the HER-2/*neu* gene in human breast cancer results in measurable changes in molecules associated with cell-cell, cell-stroma and cell-matrix interactions. These observations may account, in part, for the changes in metastatic potential demonstrated by these cells.

B) HER-2/*neu* expression levels can directly effect the expression levels of other members of the type 1 receptor tyrosine kinases, i.e. HER-1, HER-3 and HER-4. This is critical in that it may directly effect the signaling pathway in which HER-2 plays a significant role.

C) We have also demonstrated that combinations of several other chemotherapeutic agents, with anti HER-2/*neu* antibody can result in synergistic or additive therapeutic effects. This is of potential importance in developing new clinical strategies for HER-2/*neu* overexpressing breast cancers. These data were generated with both in vitro and in vivo preclinical models adding credence to their clinical applicability.

D) Perhaps one of the most exciting findings from this year's work has been the expansion and further development of studies on the interaction between HER-2/*neu* expression and the estrogen receptor. These studies are likely to be exploitable in the clinic since anti- HER-2/*neu* antibody can reverse HER-2/*neu* induced tamoxifen resistance. This area will be pursued actively in the coming funding period.